KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS-ENCODED CYCLIN, K-CYCLIN ENHANCES NF-KAPPAB-DEPENDENT TRANSCRIPTION AND INTERACTS WITH LATENCY-ASSOCIATED NUCLEAR ANTIGEN IN VIRAL AND NON-VIRALLY INFECTED CELLS

By

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To my mother, Norma Jean Duell, my first and best teacher, who gave me life and taught me how to live it. She prepared me for a day when I will have to live without her, although I cannot imagine the inevitable. She is Glenda the Good Witch to my Dorothy, and just as beautiful in my eyes as Lena Horne. Her words have guided me through Oz and eased me down the yellow brick road of life as I have looked for my own courage, brain and heart. She gave me the ruby slippers that reminded me that others add to life's journey but my service to Him and the world make my life worth going through that journey. My home is anywhere I have family, anywhere my heart can grow and flower. She believed in me when I did not believe in myself and taught me to trust totally and carefully: in myself, in others, but mostly in God.

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Kaposi's sarcoma ..... KS
Kaposi's sarcoma associated herpesvirus ..... KSHV
human herpesvirus-8 ..... HHV-8
primary effusion lymphoma ..... PEL
multicentric Castlemen's disease ..... MCD
Kaposi's sarcoma-associated herpesvirus encoded-cyclin/
open reading frame 72 ..... K-cyclin
Latency-associated nuclear antigen/ open reading frame 73 ..... LANA
v-FLICE like inhibitory protein/open reading frame 71 ..... v-FLIP
Epstein-Barr Virus ..... EBV
Retinoblastoma protein ..... pRb
cyclin dependent kinase ..... cdk
deoxyribonucleic acid ..... DNA
ribonucleic acid ..... RNA
guanine cytosine ..... GC
terminal repeats ..... TR
open reading frame ..... ORF
viral-macrophage inflammatory protein ..... v-MIP
viral-G coupled protein receptor ..... v-GCPR
small viral capsid antigen ..... v-SCA
v-interleukin 6 ..... v-IL6
viral B cell lymphoma 2 ..... v-Bcl-2
viral interferon regulator factor ..... v-IRF
open reading frame 50 ..... ORF 50/Rta
nuclear factor-kappa B ..... NF- B
EBV latent membrane protein LMP-1
human T-cell leukemia virus-1 ..... HTLV-1
sodium dodecyl sulfate polyacrylamide gel ..... SDS-PAGE
growth phase 1 ..... G1
synthesis phase ..... S
growth phase 2 ..... G2
mitosis ..... M
nuclear localization sequence ..... NLS
cyclic-AMP recognition element binding protein ..... CREB
activation transcription factor 1 ..... ATF1
LANA binding site ..... LBS
recognition element ..... RE
Bacteria artificial chromosome ..... BAC
origin replication complex ..... Orc
Fas-associated protein with death domain ..... FADD
downstream core promoter element ..... DPE
TATAAA element ..... TATA
human telomerase reverse transcriptase ..... hTERT
glycogen synthase kinase-3 beta GSK-3β

E2F transcription factor family E2F
inhibitor of DNA binding-2 ..... Id2
cyclin activating kinase ..... CAK
brahma-related gene-1 ..... BRG1
histone acteyltransferase ..... HAT
human immunodefiency virus ..... HIV
arginine, any amino acid, leucine motif ..... RXL
Signal transducer and activator of transcription ..... STAT
murine herpesvirus 68 ..... MHV-68
DNA polymerase II ..... pol II
transcription factor IID ..... TFIID
methionine, arginine, alanine, isoleucine, leucine sequence ..... MRAIL
CREB-binding protein ..... CBP
cell division cycle ..... cdc

CHAPTER I

INTRODUCTION

All functions performed by the human body must simultaneously happen in each cell comprising that body. On a large scale, these processes are undertaken by groups of cells organized into organ systems. But on a much smaller scale, each cell is executing all life functions. One of these processes, growth, includes all of the tasks necessary to regulate size, function and state of the cell. Growth is driven by several factors including the interaction between cell surface receptors and their downstream signaling. For this discussion though, the preeminent level of regulation happens through the cell cycle, a procedure by which cells must pass checkpoints or meet established requirements in order to continue to grow and replicate. Viruses, living as parasites, hijack and alter cell processes and regulatory pathways for their own growth and proliferation even if detrimental to the cell. Any interference in the cell cycle, whether through genetic mutations or in this case a virus, is the principle definition of cancer--deregulated growth (Ewen et al. 1993). The following work will present established dogma on cell cycle and transcriptional regulation in the context of Primary Effusion Lymphoma (PEL) caused by Kaposi's sarcoma-associated herpesvirus (KSHV), particularly in the latent phase, with a focus on the role of the viral gene product, Kaposi's sarcoma-associated herpesvirus-encoded cyclin (K-cyclin).

Objective

The goal of this work is to characterize the interaction and functionality of the interaction between Kaposi's sarcoma herpesvirus encoded-cyclin (K-cyclin) and latency-associated nuclear antigen. More generally, a better understanding of the interaction of viral gene products through these particular related proteins is important, as we strive to understand the basis of its transforming properties. By examining the structure, function and cooperation of K-cyclin and LANA, we hope to better understand the role that viral genes play in oncogenesis and pathogenesis. There is limited data to support the role of both proteins in replication with more available to support LANA's

role in episomal maintenance. Based on the literature and unpublished data from the Browning Lab, we believe that the interaction between these viral gene products and a potential affect on transcription could explain a previously unknown association between these proteins.

Kaposi's Sarcoma-Associated Herpesvirus

The causative agent of Kaposi's sarcoma (KS), multicentric Castlemen's disease (MCD) and PEL is KSHV, also called human herpesvirus 8 (HHV-8) (Chang et al. 1994). This γ-2-herpesvirus, that like the other γ-herpesviruses--murine herpesvirus 68, herpesvirus samiri and Epstein-Barr virus (EBV) can contribute to cancer. By hijacking the host cell's own established systems, KSHV will address its goals of advancing host cell proliferation, blocking apoptosis and ensuring viral progeny. While the viral proteins can interact and phosphorylate pRb with the D-type cyclin dependent kinase (cdk) partner cdk6, they are not susceptible to the cdk inhibitors. This interruption in cell cycle regulation plays a role in its oncogenic potential (Chang et al. 1996, Cesarman et al. 1996, Moore et al. 1996, Swanton et al. 1997).

KSHV genome

The principal infected cell in KS tumors is the spindle cell, an unusual one that expresses surface markers of both endothelial cells and macrophages (Ensoli et al. 2001). The majority of KS-associated spindle cells, although containing a low copy number, are infected with KSHV and express latent proteins; but, a few of the associated inflammatory cells appear to be infected as well. As a result, the KSHV genome present in spindle cells is thought to be at the heart of viral pathogenesis (Davis et al. 1997). Most of these latently infected cells do not secrete virus. Small subsets of them express lytic genes (Staskus et al. 1997), and electron microscopy confirms that these cells are producing viral progeny (Orenstein et al. 1997). Preliminary molecular epidemiologic studies of virus derived from clinical samples reveal that subtypes cluster by geographic region and that virus repeatedly detected from the same individual does not vary (Zong et al. 1999, 2002). Superinfection with more than one strain of HHV-8, however, has also been reported (Beyari et al. 2003).

The KSHV genome is packaged into an icosahedral capsid of approximately 1200 angstroms (Trus et al. 2001) with a typical herpesvirus envelope and tegument. The linearized genome is double-stranded DNA with approximately 145 kilobases of unique coding region and 801 basepairs of GC rich sequence at either end called the terminal repeats (TR) (**Figure 1**, Russo et al. 1996). More than 80 open reading frames (ORFs) have been identified and are numbered consecutively from the left-hand side of the genome, with the genus-conserved regions designated with ORF and the frames unique to HHV-8 named K1 through K15. Methylation of the episome likely plays a role in maintaining latency, a phase identified by the lack of production of viral progeny (Chen et al. 2001). Although the precise sequence of events leading to the activation of lytic replication has not been defined, the gene product of ORF50 is necessary and sufficient to initiate the lytic phase of the HHV-8 life cycle (Gradoville et al. 2000, Deng et al. 2002).

Life Cycle: Lytic and Latent Phases

Gene expression can be characterized into groups based on function and the order of expression. The most definitive classes of proteins are those made in the latent and lytic phases of the host cell cycle. When the host cell shifts from lytic to latent phase, a number of herpesviral homolog genes become active at the same time that other genes turn dormant. For example, one of the first genes to become active upon this shift is ORF 50, a homolog of the EBV protein Rta, whose promoter can be activated by the KSHV virion alone (Lu et al. 2005).

The lytic phase of KSHV is the time of the life cycle when the host cell is producing mature virions able to infect other cells. In order for this to happen, the virus must regulate which genes are expressed and their order. The immediate early transactivators are those turned on first during the lytic phase. They are primarily responsible for regulation of gene expression and include ORF50 (RTA), K8 (K bZIP or Zta), and ORF 57, a regulator of post-translational modifications. Secondly, early genes are made and responsible for viral DNA replication and its processing. For example, viral thymidylate synthase, which is involved in nucleotide biosynthesis is transcribed at this time, as well as viral chemokines and chemokine receptors like viral-macrophage

inflammatory protein (v-MIP), viral-G coupled protein receptor (v-GCPR). The last or late genes are those involved in maturation and packaging of the virus like small viral capsid antigen (sVCA) encoded by ORF65 and (Sun et al. 1999, Zhu et al. 1999, Jenner et al. 2001, Paulose-Murphy et al. 2001).

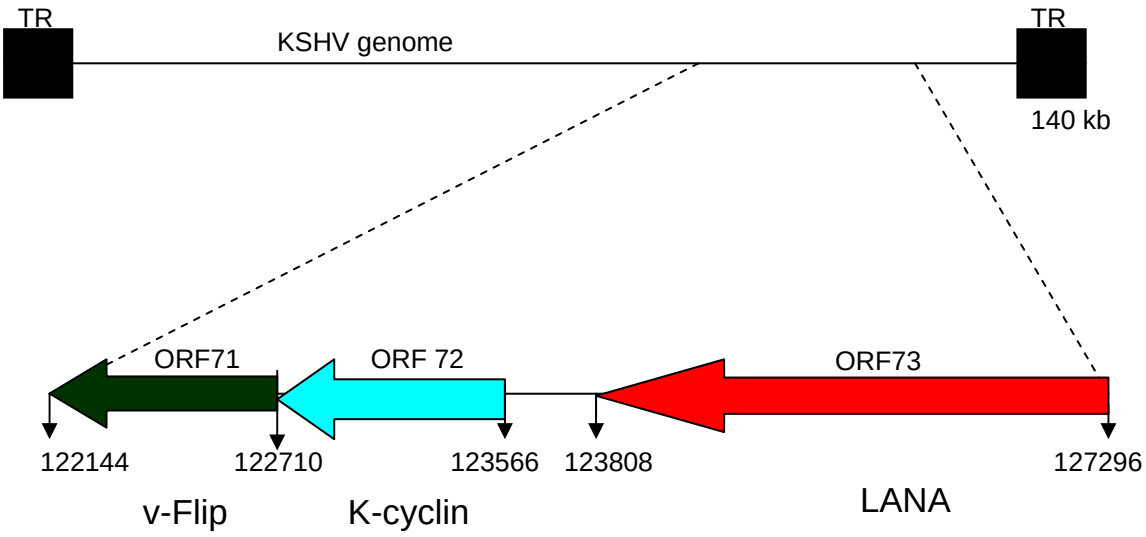

Figure 1. **Kaposi's Sarcoma-Associate Herpesvirus' Genome Encodes Three Latent Proteins Regulated by the Same Promoter**. Kaposi's sarcoma-associatedd herpesvirus (KSHV) is a DNA virus that infects human B cells. The KSHV genome is approximately 140 kilobases in length and attaches itself to genomic DNA as an episome, remaining circular and not integrated. The magnified section of DNA, shown here, encodes for three latent proteins regulated by the same promoter. ORF71, latency-associated nuclear antigen (LANA), ORF 72, viral-encoded cyclin homolog, K-cyclin, and viral-FLICE like inhibitory protein, v-FLIP are made on one transcript and then are spliced during processing for translation.

Latent Gene Products

The best characterized latent genes are clustered together and play integral roles in overriding normal cell cycle checkpoints, anchoring and directing the processing of the viral episome and blocking apoptosis. These three genes, ORF72 encoding K-cyclin, ORF73 encoding LANA, and ORF71 encoding viral Fas-ligand interleukin-1B-converting enzyme inhibitory protein, or v-FLIP, are all regulated by the same promoter, made as one message and the proteins spliced out to expression (**Figure 1,** Hu et al. 2005). K-cyclin, a cyclin D2 mimic, is the primary agent of cell cycle deregulation (Child

et al. 2001). LANA plays an integral role in establishing and maintaining the episome in the host cell (Ballestas et al. 1999). vFLIP protects cells from apoptosis by blocking the activation of the Fas death receptor pathway (Irmler et al. 1997). This inhibits the cell from tagging itself for termination by cytotoxic T-cells, even if an internal problem is identified (Djerbi et al. 1999). v-IL-6, v-Bcl-2, v-IRF are cellular homologs that promote cell proliferation and growth, induce angiogenesis, support cell survival and evade the natural immune response (Tanaka et al. 1996, Arvanitakis et al. 1997, Cheng et al. 1997, Gao et al. 1997). Virally infected cells spend the majority of their life cycle in latency, even when the viral genome itself is no longer present and so these genes are expressed in KS spindle and PEL cells and so are directly related to the cell's physical characteristics (Sun et al. 1999).

NF-κB Activation in KSHV infected Cells

NF-κB is a key pathway in the establishment of latency in KSHV infected B-cells (Krug et al. 2007). Therefore, it is not surprising that this pathway would be a vital target for one or more viral genes. The fact that all KSHV infected cell lines have the same level of NF-κB activation shows its importance in pathogenesis (Keller et al. 2000). These studies also suggest that the activation's source is similar in both cell lines and spindle cells. Because NF-κB regulates the expression of several anti-apoptotic genes like Bcl-2 and Bcl-xL, it is a common target of virally infected cells (Grimm et al. 1996, Tsukahara et al.1999). In Epstein-Barr Virus (EBV), latent membrane protein-1 (LMP-1) is the activator, in human T-cell leukemia virus type 1 (HTLV-1), tax is the culprit (Chaudhary et al. 1999, Djerbi et al. 1999, Tsukahara et al. 1999, Huang et al. 2001). In KSHV, v-FLIP has been shown to be a more efficient activator of NF-κB than cellular FLIP and is responsible for seventy percent of NF-κB in KSHV cell lines (Chaudhary et al. 1999, Guasparri et al. 2004). v-FLIP's mechanism involves using its two DED domains to block the interaction between Fas-associated protein with death domain (FADD) and pro-caspases 8 and 10, an interaction required for the signaling cascade used to trigger apoptosis (Thome et al. 1997). v-FLIP can also activate IκK complexes which can result in IL-6 expression. When v-FLIP was suppressed, one hundred percent of

activating protein-1 (AP-1) activity and seventy percent of NF-κB associated with IL-6 was abolished (Djerbi et al. 1999, Liu et al. 2002, Field et al. 2003, An et al. 2003).

Although there was some evidence to support that K1, v-GPCR and K15 played a central part in NF-κB activation in latency, Guasparri et al. eliminated that possibility by showing that v-FLIP's action is essential for the survival of infected lymphoma cells (Samananeigo et al. 2000, Pati et al. 2001, Guasparri et al. 2004). This work reiterates how viral proteins have exploited NF-κB's action for their own purposes in this case to ensure their own survival. Discussion of KSHV gene products from this point will revolve around two of the clustered latent genes, LANA and K-cyclin which will be shown to associate in the G1 phase of the cell cycle.

Latent Associated Nuclear Antigen

Latent Associated Nuclear Antigen (LANA) is a multifunctional protein that acts as a viral liaison for episomal maintenance, transcription, cell cycle regulation and DNA replication. The protein is expressed as a part of a 5.32 kilobase transcript made in the latent phase of the virus and spliced to make three different proteins (**Figure 1**). ORF71 makes viral FLICE-like inhibitory protein (v-FLIP), ORF72 makes viral cyclin D mimic (K-cyclin), and ORF73 makes LANA (Dittmer et al. 1998, Sun et al. 1999, Low et al. 2001, Kellam et al. 1997). Although the 1162 amino acid protein has a theoretical molecular mass of 135-140 kilodaltons, it actually migrates in an SDS-polyacrylamide gel (PAGE) between 220-239 kilodaltons (Renne et al. 1996). The N-terminal domain of LANA includes a nuclear localization signal (NLS), a chromosome binding and proline rich region all involved in tethering the protein to host chromatin. The highly acidic central region acts as a flexible linker region made up of repeats rich in glutamine and glutemic acid (Russo et al. 1996). The hydrophobic C-terminal domain contains a leucine zipper motif that has been shown to physically bind one LANA molecule to p53, pRb, histone H1, CREB/ATF, the LANA promoter, and itself (**Figure 2**, Fribourg et al. 1999, Gao et al. 1999, Lim et al. 2000, Radkov et al. 2000, Barbera et al. 2004, Jeong et al. 2004, Wong et al. 2005, Cai et al. 2006). In this case, the structure/function relationship is essential as it facilitates the protein's tethering ability.

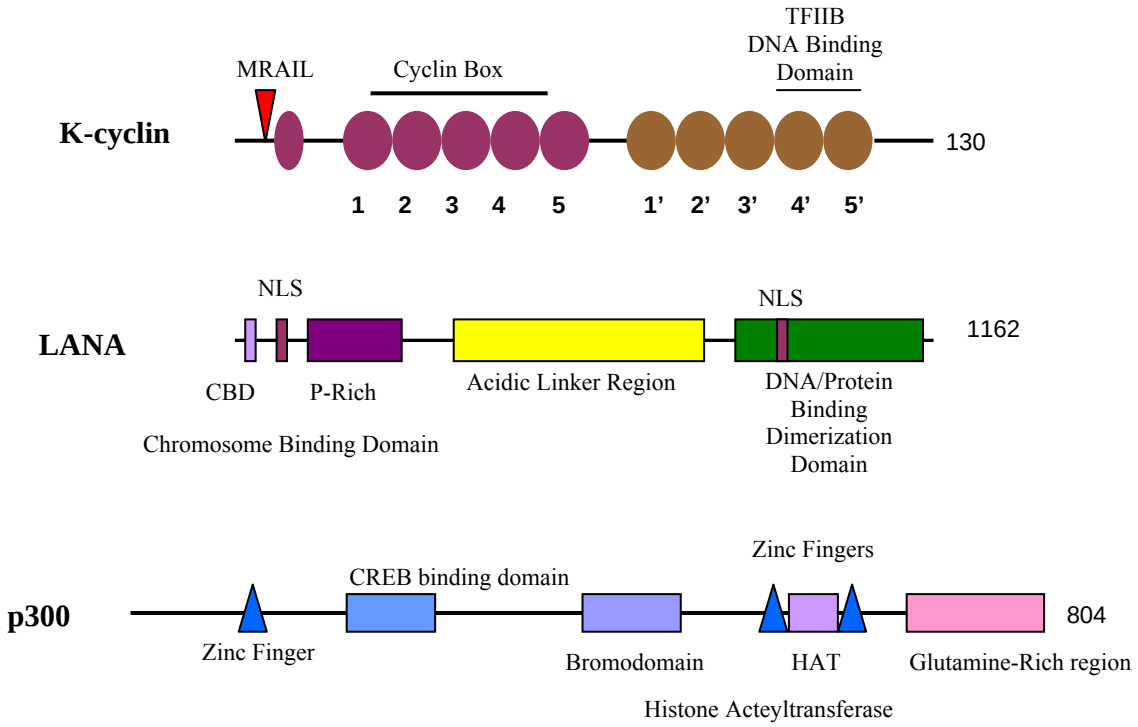

Figure 2. **K-cyclin, LANA and p300 Protein Structure**. **(A).** K-cyclin is a viral homolog of cellular D-type cyclins. It contains two alpha-helical domains, the first being the signature cyclin box, where cellular cyclins are known to interact with cyclin dependent kinases (cdks). The second alpha-helical domain is responsible for binding to transcriptional machinery like TFIIB. The protein docking site, MRAIL, which may play a role in substrate selection is located at the N-terminus beginning at position 50. **(B).** LANA is a nuclear protein known to play a significant role in episomal maintenance, DNA replication and transcription. Episomal maintenance is mediated by the chromosome binding domain (CBD) while DNA binding, dimerization and other protein binding by the C-terminal domain. The center acidic region acts as a flexible linker region to connect the protein, important for LANA's tethering of the episome to genomic DNA. **(C).** p300 is a transcriptional co-activator known to be present during NF-κB activation. The protein can bind to several other transcriptional regulators like p53, c-Jun, and c-Myc through its CRB binding domain, bromodomain and glutamine-rich regions. p300's activation ability comes from its histone acetyltransferase (HAT) activity. This allows p300 to acetylate chromatin binding histones and relax the DNA for transcriptional activity.

Episomal Maintenance

LANA is required for the replication and maintenance of the viral genome. Because the genome never inserts itself into the cellular DNA, it must attach itself in a particular way to ensure its replication and succession to daughter cells (Ballestas et al. 1999, Ballestas et al. 2001, Cotter et al. 2001, Grundhoff et al. 2003, Lim et al. 2004). LANA does this by using its N-terminus to bind to the nucleosome, in particular histone

H1, to achieve long-term persistence probably in concert with other cellular proteins (Shinohara et al. 2002). On the other end, the C-terminal part of LANA, specifically amino acids 884-1089, can bind to each other and form a dimer, while amino acids 996-1139 bind directly to the TR region of the viral DNA (Schwam et al. 2000). The two LANA binding sites (LBS1/2) are 22 basepairs apart in the TR region and both contribute to repress transcription and facilitate DNA replication (Garber et al. 2002). When a virus was created without LANA (BAC36 ΔLANA), the episome was not maintained and the cells were virus free in two weeks (Ye et al. 2004). In addition, when small hairpin RNA was used to knock down expression of the whole latent cluster of proteins, there were reduced numbers of episomes per cell. A deletion of amino acids 5-22 of the protein were sufficient to ablate episomal maintenance but could be re-established with the addition of the histone H1 protein (Shinohara et al. 2002).

DNA Replication

While the literature clearly supports LANA's role in episomal maintenance, several groups have investigated a role in DNA replication separately. The origin replication complex proteins (Orcs 1-6) must assemble in the proper order to begin the process and subsequent cascade of cdc and MDM proteins required for licensing initiation of DNA replication (Xua et al. 1998). EBNA1, another viral protein involved in DNA replication, sets a clear precedent for this role as it and LANA both co-immunoprecipitate from nuclear extracts with members of the ORC protein complex (Dhar et al. 2001, Lim et al. 2002). In LANA, this association to the ORC proteins occurs through the C-termini. The section of DNA is the same region containing the LANA binding sites implicated in episomal maintenance, noting that a single LBS is sufficient to support plasmid replication in transient assays (Hu et al. 2002). Furthermore, LANA can induce a strong bend in the DNA. This bend is the greatest when both binding sites are occupied, turning it 110 degrees, but either binding site alone will open the DNA toward the major groove 57 degrees. This reordering of the DNA could facilitate the assembly of the pre-replication complex or its licensing factors (Wong et al. 2005).

Transcriptional Regulation

While its primary role involves episomal maintenance with recent work supporting its role in DNA replication, LANA also functions as a transcriptional regulator. While it can activate EBV and human immunodefiency virus (HIV) promoters, its mechanisms of activation have been characterized using a variety of cellular and viral promoters, including its own (Groves et al. 2001, Lim et al. 2001, Renne et al. 2001, Garber et al. 2002, Verma et al. 2004). The LANA promoter, which contains a DPE and TATA box, is defined as the region between +11 and +271 upstream of the mRNA start site. This structure is rare in eukaryotes, in that most promoters have either one or the other site as their function is usually the same. In this case, neither site is sufficient to support the basic transcriptional machinery and so needs other downstream factors. The GC box/Sp1 binding site at -29 is additionally required as additional support for LANA regulation of its own promoter (Garber et al. 2001, Jeong et al. 2004). In human telomerase reverse transcriptase (hTERT) activation, LANA does not bind to DNA, but instead interacts with the Sp1 protein and acts as a co-activator (An et al. 2002). In a similar way, LANA acts as a transcriptional co-activator of c-Jun and specifically binds to the AP-1 response element (RE) to induce IL-6 transcription (An et al. 2002). This may be significant because IL-6 is NF-κB-dependent and was reported to be an essential growth factor for the KSHV related diseases (An et al. 2004). LANA is also known to stabilize β-catenin in the cytoplasm by binding to GSK-3β, interact with STAT3 and modulate TGF-β signaling and associate with Jkappa, a Notch pathway protein, to repress the viral protein, RTA (Fujimoro et al. 2005, Lu et al. 2005, Muromoto et al. 2006). This repression in turn causes a "de-repression" of LANA, as RTA limits the activity of the LANA promoter (Jeong et al. 2004). When LANA is tethered to constitutively active promoters, it interacts with itself, heteroprotein 1, mSin3, CREB/ATF and CREB binding protein as well as CIR to inhibit constitutively active promoters *in vitro* (Lim et al. 2003).

Cell Cycle Regulation

These transcriptional properties also relate to the cell cycle as LANA can modulate p53 expression as well as binding directly to pRb and releasing the E2F

transcription factors (**Figure 3**, Fribourg et al. 2000). These cells are protected from p16 induced cell cycle arrest by LANA's interaction with BRD4 and its subsequent activation of the cyclin E promoter (An et al. 2005, Viejo-Borbolla et al. 2005, Ottinger et al. 2006).

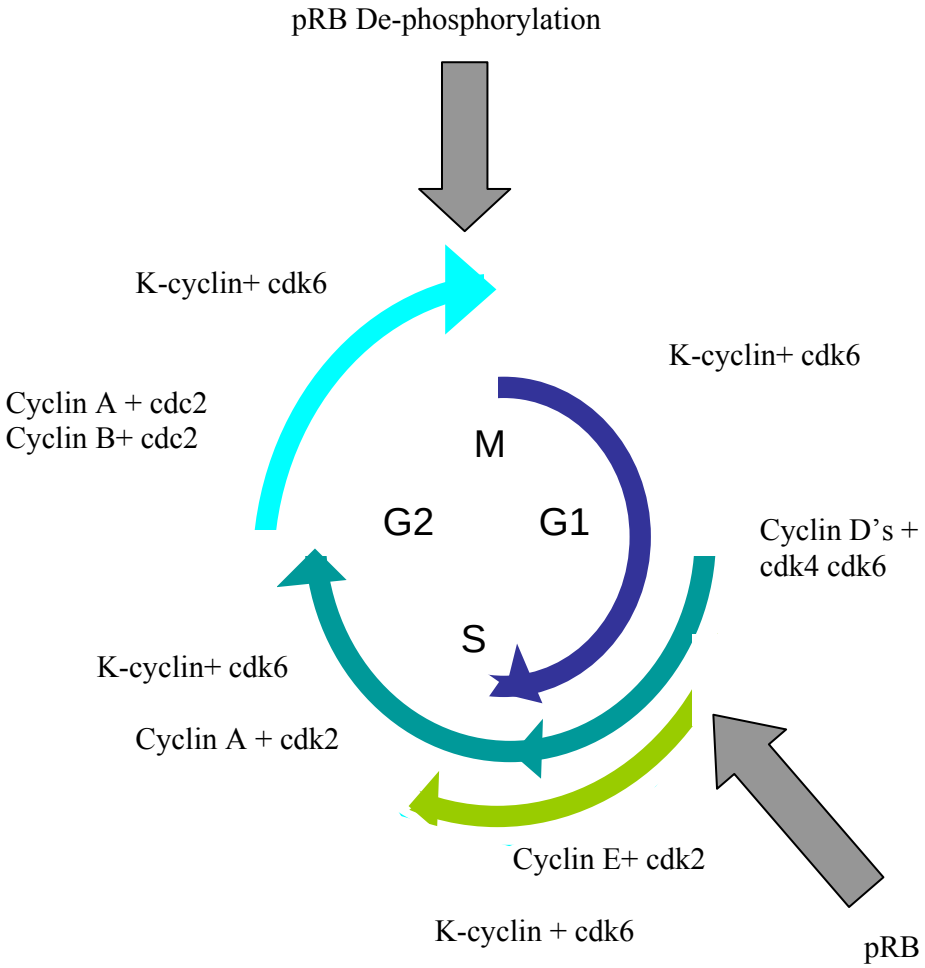

Figure 3. **K-cyclin is Constituitively Active throughout the Cell Cycle**. The cell cycle is tightly regulated by cyclin proteins. These proteins are must be activated by the cyclin activating kinase (CAK), and are present only during certain phases when they bind to certain cyclin dependent kinases (cdks) and have their action. When their work is complete, they can be repressed by cdk inhibitors and are immediately targeted for degradation. Progression through the cell cycle is based on the phosphorylation and de-phosphorylation of the pRb protein by the cyclins. This checkpoint verifies the complete working order of the cell before it is allowed to replicate and divide. If the cell cannot pass the checkpoint or is insuitable in replication, it will be targeted for apoptosis. In KSHV infected cells, K-cyclin dysregulates this system. K-cyclin does not need CAK phosphorylation to become active. It can bind to cdks 2, 4 and 6, and act in different phases of the cell cycle. K-cyclin can also phosphorylate Rb and evade this cell cycle checkpoint. Additionally, K-cyclin/cdk6 complexes are constitutively active throughout the cell cycle and can give unsuitable cells license to replicate.

In this way, LANA has cemented its role as a fundamental aspect of KSHV latency through its role of modulating several cell and viral processes including episomal maintenance, DNA replication, transcription, and cell cycle regulation.

Kaposi's Sarcoma Associated Herpesvirus Encoded-Cyclin, K-cyclin

The primary function of the cell is to grow and replicate in an orderly fashion. This work happens under several strictly regulated layers united under the overarching theory of the cell cycle. It divides the time between the formation of two daughter cells called mitosis and the next set of daughter cells. Therefore, it is the acknowledgement of the mitosis signal and preparation for that mitosis. Transitions between phases of the cell cycle are regulated by the activity of cyclin proteins and cyclin dependent kinases (cdks). Cyclins D/E mediate the progression from G1 to S phase. Cyclins A/B mediate the transition between S to G2 phases and then from G2 to the mitosis or M phase. These cyclins bind to their appropriate cdk partner, cdks 1, with cyclin B, cdk 2, with cyclin E and cdks 4, and 6 with D-type cyclins (**Figure 3**, Zhang et al. 2000, Wu et al. 2001).

Cell Cycle Regulation

The major responsibility of cyclin D/cdk complexes are to activate the cell cycle checkpoint between G1 and S phases, with cyclin B/cdc2 complexes responsible for stimulation of dephosphorylation of pRb for the exit from mitosis. This checkpoint is the hyperphosphorylation and subsequent disassociation of pRb and its binding partner E2F. When pRb is hypophosphorylated, it directly represses the cyclin A promoter, thereby blocking S-phase progression (Ewen et al. 1993, Zhang et al. 2000). When E2F is released, cyclin A expression is allowed. The binding also directly blocks the active site of E2F and represses its transcriptional activity (Helin et al. 1993). E2F1-6 can dimerize with DP1-2 proteins in all possible combinations and act as potent activators for proliferation factors (Wu et al. 2001).

K-cyclin, like other viral cyclins, is a cellular cyclin mimic made by KSHV to deregulate the host's cell cycle and impose its own control of cellular pathways. The protein is expressed as a 5.32 kilobase transcript made in the latent phase of the virus and spliced to make three different proteins, ORF71 viral FLICE-like inhibitory protein (v-

FLIP), ORF72 viral cyclin D mimic (K-cyclin), and ORF73 LANA (**Figure 1**, Kellam et al.1997, Dittmer et al. 1998, van Dyk et al 1999). K-cyclin, shares thirty-one percent amino acid structure and fifty-three percent similarity with cellular D-type cyclins (Cannell et al. 1999). While cyclin D2 binds to cdks 4 and 6, K-cyclin binds to those and additionally cdk2, cyclin E and A's standard kinase partner (Jung et al. 1994, Godden-Kent et al. 1997, Li et al. 1997). Cyclin D2 is inhibited by p21 and p27, while no cdk inhibitors by K-cyclin. For example, in the case of p27, its inhibition is interrupted by K-cyclin and its ability to phosphorylate p27's amino acids 10 and 187 and decrease overall protein stability (**Figure 4**, Swanton et al. 1997, Sarek et al. 2006). Cyclin D2 furthermore, has only one known phosphorylation target, pRb, whereas K-cyclin's phosphorylation targets include pRb, p27, histone H1, Bcl-2, inhibitor of DNA binding-2 (Id2), cdc25a and p300 (**Figure 4**, Ojala et al. 2000, Laman et al. 2001, Sarek et al. 2006, unpublished data). While cellular cyclins/cdk complexes are regulated by the phosphorylation and activation by the cyclin activating kinase protein (CAK), K-cyclin is active even when not phosphorylated by CAK, making it less dependent on host-cell interactions for its activity (Child et al. 2001). Additionally, our studies in KSHV-infected cells show that the half-life of K-cyclin is greater than that of cellular cyclin D2 (half-life of ~6-8 hours vs. ~0.5 hours respectively). The decreased turnover of K-cyclin results in constitutive activation of K-cyclin/cdk6 complexes throughout the cell cycle. K-cyclin is both cytoplasmic and nuclear but most important, K-cyclin/cdk6 complexes are kinase active throughout the cell cycle (**Figure 3**, Davis et al. 1997, Van Dross et al. 2005). This uncontrolled K-cyclin/cdk6 kinase activity may be essential for maintaining viral latency and ultimately, contribute to the oncogenic nature of KSHV.

In addition to its work directly with cell cycle regulation proteins, K-cyclin also can directly trigger the initiation of DNA synthesis. K-cyclin /cdk6 complexes can phosphorylate Orc1, implicating K-cyclin in DNA replication and possibly licensing of the pre-replication complex (**Figure 4**, Laman et al. 2001). The literature supports this finding in that cyclin A/cdk2 can interact with the ORC complex by recruiting it to the origin of replication (Xua et al. 1998). K-cyclin deregulates the cell cycle by regulating pRb at both cell checkpoints.

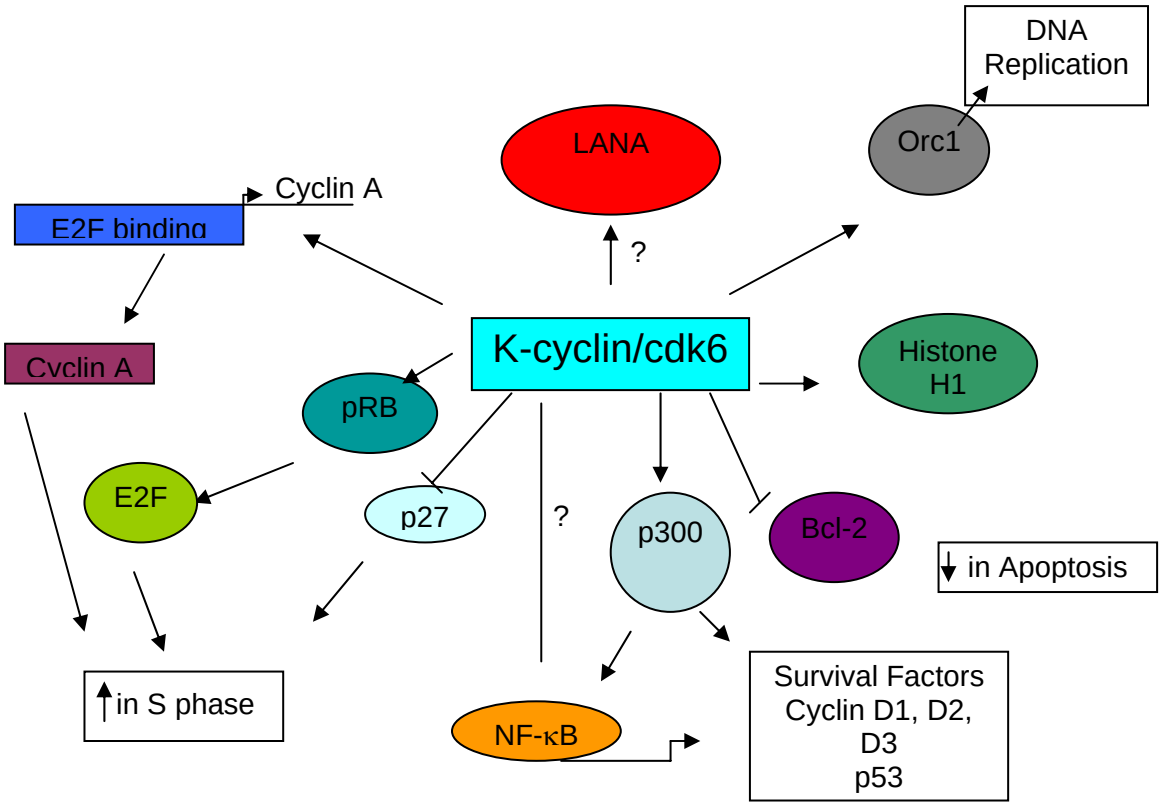

Figure 4. **K-cyclin/cdk6 Complexes Interacting Partners and Phosphorylation Targets.** The figure is an illustration of some of the known K-cyclin interacting proteins and their implications in cell cycle, apoptosis, DNA replication and transcription. In its role to deregulate cell cycle, K-cyclin/cdk6 compleses can phosphorylate pRb, releasing E2F and advancing cells through the first cell cycle checkpoint and into S phase. Phosphorylation of p27 can stop its inhibitory effect on cell cycle and cause it to be degraded. Then, K-cyclin/cdk6 complexes can interact with the E2F binding site in and upregulating Cyclin A transcription, another promoting factor for S phase. Additionally, phosphorylation target Orc1 is an integral part of licensing for DNA replication. Bcl-2 is phosphorylated by K-cyclin/cdk6 complexes and inhibits the activation of the apoptotic pathway. K-cyclin's involvement in the transcriptional pathway, in addition to cyclin A, revolves around NF-κB and p300. K-cyclin/cdk6 complexes can phosphorylate p300 and by an unknown mechanism regulate the NF-κB transcription factor family. This activation can upregulate survival factors like the D-type cyclins and anti-apoptotic genes like Bcl-xL. Finally, we have shown that K-cyclin can interact with LANA and we theorize that it modulates LANA's role as a transcription factor.

As previously stated, K-cyclin/cdk complexes are not dependent on CAK activity and so are less regulated by mitogenic and other cellular factors. Although the mechanism by which K-cyclin affects mitotic exit is unknown, K-cyclin expressing cells continually undergo DNA synthesis and nuclear division but have problems with cytokenesis (Verschuren et al. 2002). With its phosphorylation of pRb to bypass

checkpoints, no regulation from the cyclin dependant kinase inhibitors and constitutive activation throughout the cell cycle with at least cdk6, K-cyclin can continuously accelerate its host cell through the phases of the cycle influencing growth, proliferation, and replication.

Implications in Transcription

There is one published example of K-cyclin directly regulating the transcription of another protein, cyclin A. Although it does not phosphorylate pocket protein p107 or interact directly with E2F, transcription is facilitated through the E2F binding site in the cyclin A promoter and is dependent on cdk6 activity (**Figure 4**, Duro et al. 1999). The similarities between K-cyclin and cellular cyclin/cdk complexes suggest that K-cyclin may be a transcriptional regulator in other cases as well. Chromatin is a tightly packed complex of DNA with histone proteins that protect DNA. The expression of cellular genes is determined in part by the structural organization of DNA in the chromatin. Gene activation requires the unfolding of sections of chromatin to permit the binding of factors comprising the transcriptional machinery. This unfolding of chromatin allows for its separation into two types, euchromatin or that which is transcriptionally active, comprising ~5-10% of all DNA, and the rest unavailable for transcription, heterochromatin (Bloom, 1978). The euchromatin is depleted of histone H1 and is enriched in hyperacetylated core histones necessary for chromatin unfolding as well as transcriptional activators, transcriptional co-activator proteins such as p300/CBP and other histone acetyltransferase proteins, and the ATP-dependent chromatin remodeling proteins, BRG1 and Brm (Komaiko et al. 1985, Hebbes et al. 1988, Oliva et al. 1990, Boyes et al.1998, Goodman et al. 2000, Vignali et al. 2000).

NF-κB-dependent Transcription

Studies show that the primary effusion lymphoma (PEL) cell lines and clinical KSHV infected PEL biopsy specimens contain constitutively activated NF-κB (Keller et al. 2000, Krug et al. 2007). This activation is important in producing cytokines and is essential for establishing latency in KSHV infected cells (Libermann et al. 1990, Guasparri et al. 2004). Furthermore, NF-κB target genes include anti-apoptotic genes

like Bcl-2 and Bcl-xL which ensure cell survival (Thome et al. 1997, Chaudhary et al. 1999, Guasparri et al. 2004). Seventy percent of IL-6 expression can be attributed directly to v-FLIP, a major source of NF-κB activation (Chaudhary et al. 1999). But unpublished data from the Browning lab identified a novel substrate of K-cyclin/cdk6 complexes in KSHV-infected cells, the NF-κB transcriptional co-activator p300/CBP.

The NF-κB protein family is a group of transcription factors that regulate the cell growth and survival, inflammatory and immune response. The transcriptional activator's target genes include IL-6, c-FLIP, Bcl-xL, Bcl-2, p53 and cyclins D1, D2 and D3 (Libermann et al. 1990, Grimm et al. 1996,Wang et al. 1996, Zhong et al. 1998, Chen et al. 1999, Guttridge et al. 1999, Tsukahara et al.1999, Catz et al. 2001, Kruez et al. 2001, Huang et al. 2004, Schumn et al. 2006). There are seven different NF-κB subunits: Rel A/p65, p105 and its splicing product, p50, p100 and its splicing product p52, c-Rel and Rel B that combine in a number of ways. Rel A/p65, c-Rel, and Rel B, contain transactivation domains but p50 and p52 do not (Nabel et al. 1987, Libermann et al. 1990, Beg et al. 1993). Their common domain is a rel homology domain that can mediate DNA binding, dimerization and interaction with the IκB family of NF-κB inhibitors. These nuclear factors are regulated by sequestration in the cytoplasm. NF-κB is the product of a signaling cascade that results in the subunits' translocation to the nucleus. Once there, NF-κB subunits can interact with CBP/p300 to effect transcription (**Figure 5**, Nabel et al. 1987, Murcurio et al. 1992, Beg et al. 1993, Dallas et al. 1993, Kwok et al. 1994, 1996, Lill et al. 1997, Perkins et al 1997, Zhong et al. 2002).

The ability of p300 to activate transcription is dependent on its histone acetyltransferase (HAT) domain. Acetylation of the histones associated to the promoter or other enhancer regions of the genes causes activation by chromatin remodeling. After remodeling it can recruit and assemble proteins like NF-κB, p53, c-myc, c-Jun, c-Fos and viral proteins E2F and Human Immunodefiency Virus (HIV) Tat (Bannister et al. 1995, Eckner et al. 1996, Ogryzko et al. 1996, Avantaggiati et al. 1997, Scholnick et al. 1997) One NF-κB subunit that interacts with p300 is p65, in an area separate from the one that interacts with cyclin/cdk complexes. This interaction and subsequent acetylation

regulates the strength and length of time NF-κB is present in the nucleus (Perkins et al. 1997, Goodman et al. 2000).

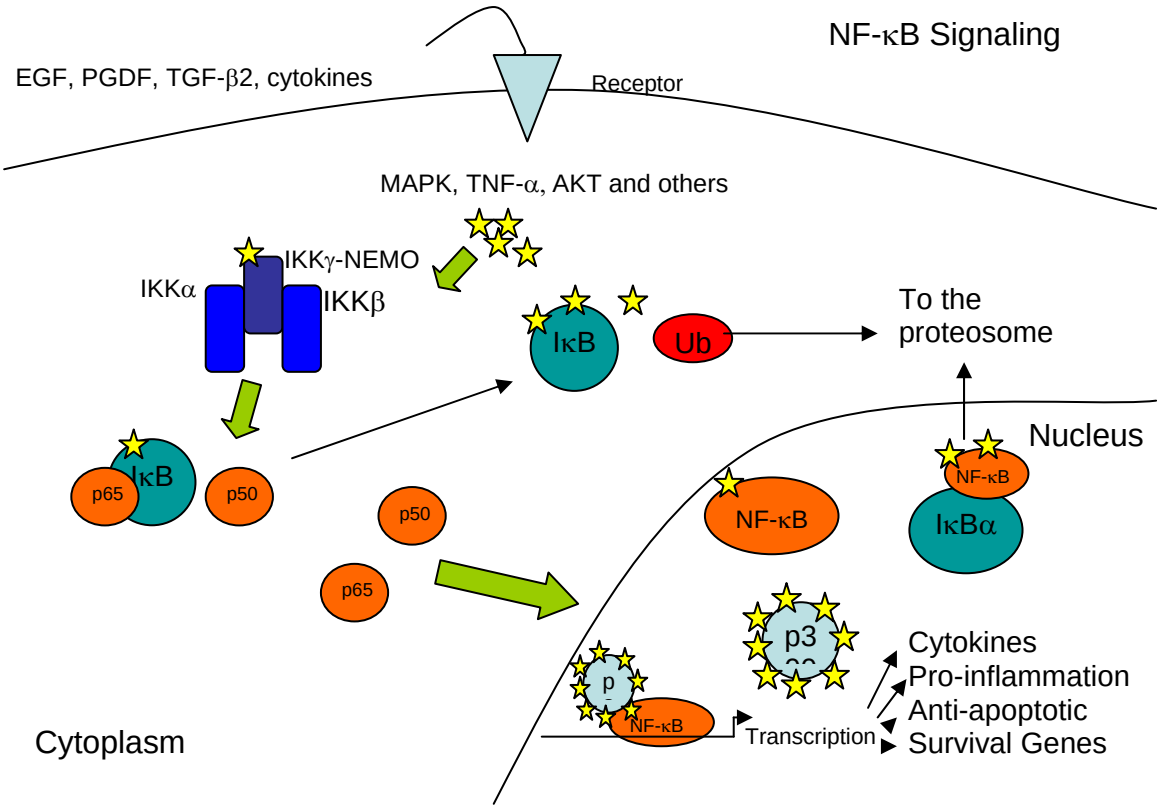

Figure 5. **NF-κB Signaling Cascade**. Extracellular ligands like epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) can activate receptors along the cell surface and begin the MAP kinase, or AKT cytoplasmic signaling cascade. The cascade finally winds up phosphorylating IKK-γ (NEMO) and releasing IκB kinase. IκB kinase will then phosphorylate IκB and subsequently release the NF-κB subunits, in this case represented by p50 and p65. Once they are released, the subunits transclocate to the nucleus. There, the subunits join other co-activators like p300 and binding to the NF-κB recognition element the DNA of their target genes. When their work is done, they will be ubiquitinated and degraded by the proteosome. NF-κB is a crucial target for viral proteins due to its wide variety of target genes that include cytokines, anti-apoptotic genes and survival factors.

The interaction with p300 can have two effects. The first is to bind with other proteins to specific DNA recognition elements and repress transcription. For example, Daxx can bind to p65 and prevent its association and therefore acetylation by p300,

repressing NF-κB (Park et al. 2007). The second is to bind to p21WAF1/CIP1 at its CRD1 domain and de-repress or activate transcription (Snowden et al. 2000). Although K-cyclin is not inhibited by p21 WAF1/CIP1, it blocks the action of p21 by phosphorylating it on serine 130 (Jarviluoma et al. 2006). This is an excellent example of how cell cycle proteins can influence the regulation of growth factors, stress response genes, cytokines, genes related to immunity, and other survival factors (Perkins et al. 1997, Richmond 2002). Moreover, unpublished data from the Browning Lab shows that K-cyclin/cdk6 complexes can phosphorylate *in vivo* and *in vitro* transcribed and translated p300 and together with p300 synergistically activate NF-κB -dependent transcription. These findings suggest that K-cyclin/cdk6 complexes could be a contributing factor for constitutive activation of NF-κB in KSHV-infected cells. This activation may act in concert with LANA activation through the AP-1 response element to increase IL-6 (An et al. 2003). Whether K-cyclin can interact in other parts of the signaling pathway in either the nucleus or cytoplasm is unknown.

K-cyclin contains a protein docking site near the amino terminus, termed the MRAIL motif. This site, identified in cyclin A, allows cellular cyclins to interact with proteins containing RXL motifs, like cyclin A/cdk2 phosphorylation targets p107 and p130 (Schulman et al.1998). Several cellular and viral cyclins have this conserved protein docking site (**Table 1**) which may mediate the selection, binding and subsequent phosphorylation of kinase substrates. These corresponding proteins can be divided into two categories: those that are cell cycle related like the E2F proteins, p107, p130, p27, pRb (**Table 3**), DNA replication related Orc1, and Orc6 (**Table 4**) and those that are transcriptional regulators like p300, CBP, and P/CAF (**Table 2**, Wilmes et al. 2004). The RXL motifs in p300 were of particular interest as we also identified potential phosphorylation sites that fit the sequence for cdk phosphorylation targets. At least one novel K-cyclin/cdk6 target, caldesmon-1, an actin and calmodulin binding protein, was identified phosphorylated at four serine/threonine and proline motifs. Caldesmon is known to be phosphorylated by cdk1 *in vivo*, while this work shows that Cyclin A and E/cdk2 complexes can also phosphorylate it *in vitro*. The caldesmon kinase-targeted sites, while similar, are not exactly the same for the different kinases. This suggests that there is another indicator for substrate specificity. Cuomo et al. proposes the

serine/threonine and then proline motif as the target for cdk6 (Cuomo et al. 2005). Subsequently, we have identified several serine/threonine proline motifs in potential interaction proteins (**Tables 2B, 3B, 4B**). Substantiating their theory, there are six Cy or RXL motifs and at least forty serine/threonine proline motifs in p300 and other K-cyclin potential interactors (**Table 2A, 4A, 6A**).

Table 1. **MRAIL motif is conserved in both Cellular and Viral Cyclins**. Acompliation of the N-terminal ends of viral and cellular cyclins, beginning atposition 50. K-cyclin is produced by Kaposi's Sarcoma Associated Herpesvirus.V-cyclin is herpesvirus saimiri, M-cyclin is murine herpesvirus 68. Cyclins D1,D2, D3, E and A are cellular.

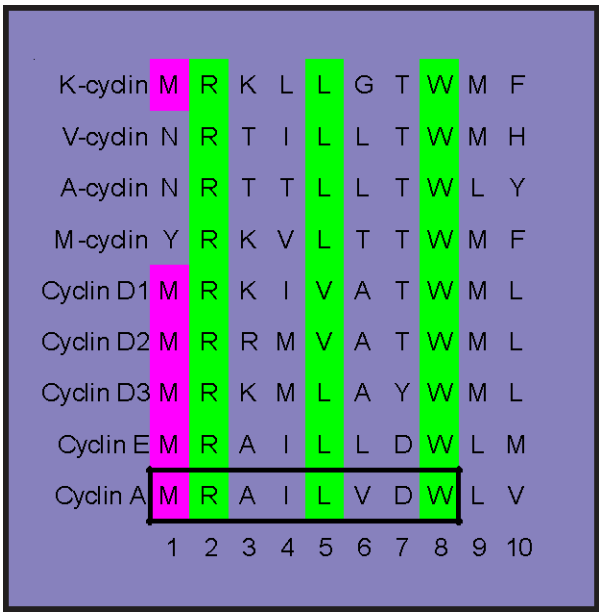

Table 2. **Potential Protein Binding and Phosphorylation Motifs in DNA replication Proteins. (A)** A survey of the amino acid sequence of several DNA replication proteins show several RXL motifs that could act as binding sites to the corresponding MRAIL protein K-cyclin and its kinase partner, cdk6. **(B)** The same proteins when scanned for potential phosphorylation sites serine or threonine and proline. There are several sites in each protein that could be subject to phosphorylation by K-cyclin/cdk complexes or other kinases. These sites suggest that through the MRAIL-RXL motifs, K-cyclin may play a role in DNA replication.

A

Protein	NCBI ID#	# of RXL motifs	Amino Acid Position #
Orc 1	NP 04144	7	24, 90, 154, 396, 441, 476, 830
Orc 2	NP 006181	4	273, 320, 482, 493
Orc 3	NP 862820	3	251, 366, 461
Orc 4	NP 002543	2	116, 277
Orc 5	NP 002544	3	70, 251, 361
Orc 6	NP 055136	1	99

B

Protein	No. of S/TP motifs	Amino Acid Position
Orc 1	10	199,203, 224, 258, 273, 286, 311, 336, 375, 610
Orc 2	5	116, 226, 280, 452, 485
Orc 3	2	212, 255
Orc 4	2	32, 185
Orc 5	0	N/A
Orc 6	1	195

Table 3. **Potential Protein Binding and Phosphorylation Motifs in Transcriptional Regulators. (A)** A survey of the amino acid sequence of several NF-┤ B subunits show several RXL motifs that could act as binding sites to the corresponding MRAIL protein K-cyclin and its kinase partner, cdk6. Three proteins p100 and its splice variant, p52 and Rel-B do not have RXL proteins. The NF-┤ B subunits act as a pair though, so interaction with only one subunit may be necessary. At least one of those proteins p52, does not have a DNA- binding domain and so must bind with another subunit that contains one. **(B)** The same proteins when scanned for potential phosphorylation sites serine or threonine and proline. There are several sites in each protein that can be subject to phosphorylation by K-cyclin/cdk complexes or other kinases. These sites suggest that through the MRAILRXL motifs, K-cyclin could interact and activate NF-┤ B activation.

A

Protein	NCBI ID#	# of RXL motifs	Amino Acid Position #
p300	Q 09472	6	335, 1104, 1166, 1731, 2036, 2262
P105 (p50)	NP 003989	5	287, 330, 661, 971,1035
p100 (p52)	NM 002502	0	N/A
C-Rel	X 75042	4	98, 378, 613, 631
Rel-B	NM 006509	0	N/A
Rel A/p65	M 62399	3	134, 191, 288, 336

B

Protein	No. of S/TP motifs	Amino Acid Position
p300	51	20, 90, 111, 124, 133, 156, 229, 255, 284, 499, 523, 730, 805, 831, 833, 839, 841, 845, 865, 885, 887, 926, 938, 1038, 1095, 1104, 1166, 1171, 1726, 1849, 1851, 1854, 1857, 1865, 1868, 1878, 1916, 1919,2039, 2056, 2060, 2162, 2271, 2279, 2291, 2309, 2315, 2326, 2328, 2336, 2341, 2346, 2356
p105 (p50)	8	402, 516, 685, 811, 865, 951, 994, 1008
p100 (p52)	3	322, 387, 391
C-Rel	3	346, 490, 519, 534
Rel-B	5	37, 103, 116, 217, 363
Rel A/p65	2	164, 236

Table 4. **Potential Protein Binding and Phosphorylation Motifs in Cell Cycle Proteins. (A)** A survey of the amino acid sequence of several cell cycle proteins show several RXL motifs that could act as binding sites to the corresponding MRAIL protein K-cyclin and its kinas partner, cdk6. **(B)** The same proteins when scanned for potential phosphorylation sites serine or threonine and proline. There are several sites in each protein that could be subject to phosphorylation by K-cyclin/cdk complexes or other kinases. These sites suggest that through the MRAIL-RXL motifs, K-cyclin could influence cell cycle in other ways.

A

Protein	NCBI ID#	# of RXL motifs	Amino Acid Position #
E2F-1	Q0194	5	26, 137, 211, 232, 422
E2F-2	NP 004082	3	6, 66, 77
E2F-3	NP 001940	2	36, 134
pRB	NP 000312	4	150, 668, 698, 741
p107	AAH32247	5	89, 481, 658, 802, 838
p130	NP 005602	4	680, 852, 868, 1028
p53	PO4637	1	306

B

Protein	# of S/TP motifs	Amino Acid Position
E2F-1	9	68, 75, 121, 311, 332, 337, 375, 403, 433
E2F-2	15	15, 23, 34, 46, 66, 104, 116, 120, 123, 312, 327, 355, 391, 398, 404
E2F-3	9	41, 102, 163, 169, 172, 197, 359, 397, 402
pRB	15	5, 230, 249, 252, 356, 567, 608, 612, 780, 788, 795, 807, 810, 820, 827
p107	16	332, 340, 369, 385, 515, 615, 640, 650, 749, 762, 915, 964, 975, 987, 997, 1009
p130	23	8, 42, 401, 413, 417, 603, 642, 662, 672, 688, 694, 720, 811, 952, 966, 986, 1035, 1044, 1059, 1067, 1080, 1097, 1112
p53	5	33, 46, 81, 137, 315

Cyclins as Transcriptional Regulators

Cellular cyclins, in addition to their main function, have emerging roles in the transcriptional regulation of proteins as well. Cyclin D1 is recruited to the p21WAF1 promoter by the STAT3-NcoA complex. This interaction hinders the recruitment of CBP and RNA polymerase II, thereby inhibiting p21 transcription (Bienvenu et al. 2005). Cyclin D1 also binds to the histone acetyltransferase, p300/CREB-binding protein-associated protein (P/CAF) to potentiate its activity with the estrogen receptor and affect its downstream targets (McMahon et al. 1999). Cyclin E can associate with the androgen receptor and mediate its activity with the association of cdk2 (Yamamoto et al. 2000). Cyclin A can interact with and phosphorylate Sp1 in a promoter specific fashion (Fojas de Borja et al. 2001, Haidweger et al. 2001). Finally, cyclins E and A can both regulate transcription when tethered to the LexA DNA-binding protein. They have opposite actions though, with cyclin E activating and cyclin A repressing transcription (Rottman et al. 2005, Santaguida et al. 2005).

Viral cyclins, too, have been shown to be involved in transcriptional regulation. The walleye fish retroviral cyclin, which shares 13% identity and 24% similarity with K-cyclin (LaPierre et al. 1999), has been shown to regulate gene expression through its interactions with RNA pol II and the Mediator complex (Quackenbush et al. 1997). MHV68 cyclin has been shown to have oncogenic properties through its ability to regulate T-cell development and cell cycle progression (van Dyk et al. 2000). Therefore, it is quite feasible that K-cyclin may behave in a similar manner as other viral cyclins in tumor development.

Hypothesis

Summarized here are unpublished data that suggest that K-cyclin is a transcription factor. First, the Browning lab has shown that K-cyclin associates with the transcriptionally active S1 fraction of chromatin along with other proteins like p300, polymerase II and transcription factor IID (TFIID) known to be involved in transcription. Second, in vitro kinase assays using K-cyclin and cdk6 proteins from transfected SF9 cells shows that these complexes can phosphorylate p300. Furthermore, we have identified protein-docking sites available in both K-cyclin and p300 that could mediate an

association between the proteins and p300 putative phosphorylation sites. Next, K-cyclin has been shown to activate NF-κB dependent transcription in reporter assays, is synergistic with p300 and is cdk dependent. Lastly, we have identified a novel association between K-cyclin and LANA, a multifunctional viral protein involved in transcription by interacting directly with DNA and through protein-protein interactions with other transcription factors like AP-1, Sp1 and pRb. These findings suggest that K-cyclin is a transcriptional regulator and that these may play a role in the signature NF-κB activation found in KSHV-infected cells.

Overview

In this work, we present the data that K-cyclin/cdk6 kinase complexes regulate NF-κB-dependent gene expression and this activation can be impeded by mutation of the MRAIL protein docking site in K-cyclin. As K-cyclin and LANA are both transcribed from a single promoter, are expressed at the same time in the viral life cycle and are both found in the nucleus, we sought to determine whether they physically interact. We show a novel association between K-cyclin and LANA, two viral proteins that interact in a protein complex exclusive of other viral proteins. These results suggest that K-cyclin-modulated kinases regulate transcription outside of the realm of the cell cycle, and that activation of the NF-κB pathway may contribute to the abnormal proliferative properties of KSHV-infected cells.

CHAPTER II

MATERIALS AND METHODS

Plasmids

K-cyclin and the fusion protein consisting of the DNA binding domain of the yeast transactivator, GAL4 BD-K-cyclin were subcloned into the eukaryotic expression vector, pBKCMV by PCR. Stephen Brandt (Vanderbilt University, TN) provided the p300 expression plasmid, pCDNA3-p300. The dominant negative p300 plasmid was provided by Antonio Giordano (Thomas Jefferson University, Philadelphia, PA), and Jennifer Pietenpol (Vanderbilt University, Nashville, TN) kindly provided the luciferase reporter minimal promoter containing the TATA element. The NF-κB responsive plasmid NF-κB-Luc was obtained from Stratagene (La Jolla, CA). Dominant negative cdk6 was a kind gift from Charles Sherr (St. Jude Children's Hospital, Memphis, TN). pcDNA 3.1 LANA was the kind gifts of Rolf Renne (University of Florida, Gainesville, FL).

The K-cyclin MRAIL mutants were generated as instructed by the manufacturer with the Quickchange XL kit Stratagene (La Jolla, CA). The following primers were used in a PCR reaction to change the final two bases of the specified codon. **M50A** 5'GCCCAGTAACTTACGCGCATGCGAAGTAAGAGA3' 3'TCTCTTACTTCG CATGCGCGTAAGTTACTGGCG5' **50,54A** 5' TCTCTTACTTCGCATGCGCG TAAGTTAGCGGGCACATGGATGTTT 3'AAACATCCATGTGCCCGCTAACT TACGCGCATGCGAAGTAAGAGA **50, 54, 57A** 5'TCTCTTACTTCGCA TGCGCGTAAGTTAGCGGGCACAGCGATGTTTTCAGTTTGC 3'GCAAACTGAAA ACATCGCTGTGCCCGCTAACTTACGCGCATGCGAAGTAAAGA.

Reagents

Sheep K-cyclin and sheep immunoglobulin G (IgG) antibodies were developed at Exalpha Biologicals, Inc. (Watertown, MA). Antibodies to LANA (product 13-210-100) were obtained from Applied Biologicals, Inc. and cdk6 (product C-21), orc1 (product H-

60) and cdc6 (product H-304) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Aprotinin, leupeptin, pepstatin A, DNase A and PMSF were from Sigma (St. Louis, MO). All other reagents in this study were reagent grade.

Cell Culture and Transfections

Primary Effusion Lymphoma (PEL) cell line (BC3) is KSHV-containing human cells. They were obtained from American Tissue Cell Culture (ATCC, Manassas, VA) and maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), sodium pyruvate, penicillin (100U/ml) and streptomycin (100ug/ml) Epstein-Barr virus positive, KSHV-negative Daudi cells were obtained from ATCC and maintained in RPMI 1640 medium. Cos-7L and 293H cells were obtained from ATCC and maintained in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum, 0.1mM non-essential amino acids (Mediatech, Herndon, VA), penicillin (100U/ml, Invitrogen), streptomycin (100ug/ml, Invitrogen), 2.5 M HEPES (Mediatech) and 2 M glutamine (Invitrogen). Cos-7L cells were transfected at 2.1x 10^6 with Lipofectamine 2000 (Invitrogen) or calcium phosphate in 10cm^2 dishes and were assayed for protein expression. Luciferase assays were performed using the Dual Luciferase Reporter Assay System (Promega, Madison, WI). Each experiment was performed in triplicate and each experiment independently performed at least three times.

In Vitro Transcription and Translation

In Vitro transcription and translation was performed according to the protocol by the TNT T7 and S6 Quick Coupled Transcription and Translation Reticulocyte System (Promega, Madison, WI). 1 ug of pBKCMV-K-cyclin DNA template with the indicated mutation was combined with prepared rabbit reticulocyte lysate, TNT reaction buffer, 1mM amino acid mixture without methionine, 40 units of RNAsin, the supplied Rnase inhibitor, nuclease- free water, T7 RNA polymerase and ^{35}S methionine with approximately 1 millicurie of radioactivity. The reaction was incubated at 30^o degrees for 60 minutes. One-fifth of the product was run on a 10% SDS-PAGE gel for resolution, gel dried and exposed to x-ray film at -80^{o}C for 2 hours.

Immunoprecipitations

BC3 or Cos-7L cells were lysed by manipulation with a 23 gauge needle four times in kinase lysis buffer (KLB) (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.1% Triton X100, 0.1% NP-40, 4 mM EDTA, 4 mM NaF, 0.1 mM sodium orthovanadate, 0.1% BSA, 1 mM PMSF, 2 μg/ml aprotinin, and 2 μg/ml leupeptin), or radio immunoprecipitation assay (RIPA) buffer (0.5M TRIS-HCl, pH7,4 1.5M NaCl, 2,5% deoxycholic acid, 10% NP-40, 10mM EDTA, 1mM PMSF, 1 ug/ml leupeptin, 1 μg /ml pepstatin A, 1 μg /ml apoprotin). All antibodies for immunoprecipitation were conjugated to protein G Sepharose beads (Amersham-Pharmacia, Piscataway, NJ). Cell lysates or chromatin fractions were pre-incubated with sheep IgG (Santa Cruz Biotechnologies, Santa Cruz, CA) for 1 hr at 4^0C and centrifuged. The cell lysates were centrifuged at 10,000g for thirty seconds, and the supernatant was transferred to a clean microfuge tube. The supernatants were incubated with the selected antibody overnight at 4^0C. The beads were washed three times with KLB or RIPA lysis buffer, and the buffer was discarded. The beads were resuspended in an equal volume of 4x NuPAGE LDS–loading dye (Invitrogen), heated for 10 minutes at 70^{o}C, and resolved using 10% SDS-PAGE.

Immunoblots

Proteins resolved by SDS–PAGE were transferred to Immobilon P membranes as instructed by the manufacturer (Millipore, Bedford, MA). The membrane was then incubated in a solution of TRIS-buffered saline and Tween-20 (TTBS = 0.02 M TRIS–HCl [pH 7.6], 0.14 M NaCl, and 0.05% Tween-20) blocking buffer (Sigma, St. Louis, MO) at room temperature for 1 hour. The membrane was incubated with antibodies against K-cyclin, at a 1:20,000 dilution in TTBS and Sigma blocking buffer. Membranes were then washed five times in TTBS and incubated with the appropriate secondary antibody (Amersham Biosciences Piscataway, NJ) at a 1: 10,000 dilution for 1 hour in TTBS with Sigma blocking buffer. After five 5-minute washes in TTBS, the membrane was incubated with enhanced chemiluminescence (Supersignal; Pierce) for five minutes, as described in the instruction manual. To visualize western blot protein bands, the membranes were exposed to x-ray film at room temperature and developed in a film processor.

Primary antibodies probes with LANA (rat monoclonal 1:1,000, ABI) were conducted overnight at 4^{o}, and cdk6 C-21 (mouse monoclonal 1:15,000, Santa Cruz), Orc1 H-60 (rabbit polyclonal 1:1,000), cdc6 H-304 (rabbit polyclonal 1:1000) for sixty minutes at room temperature followed by incubation with appropriate horseradish peroxide-conjugated secondary antibodies (Amersham). Transfer and immunodetection were performed as described above.

Nuclear and Cytoplasmic Cell Fractionation

Cytoplasmic extracts were prepared by washing 1 x 10^{8} BC3 cells in PBS, centrifuging cells as described above, and resuspending the cell pellet in nuclear separation media (10 mM TRIS–HCl [pH 7.5], 25 µM sodium fluoride, 5 mM magnesium chloride, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM sodium vanadate, aprotinin at 5 µg/mL, leupeptin at 5 µg/mL, pepstatin at 5 µg/mL, and Pefabloc at 150 µg/mL). Cells were allowed to swell on ice for 15 minutes and then homogenized in a dounce homogenizer with 20 strokes of pestle B. Nuclear material was removed from the cytoplasmic extract by centrifugation at 500*g* for 10 minutes at 4 °C, and the supernatant was further purified by centrifugation at 315,000*g* for 30 minutes. The supernatant from this centrifugation was the cytoplasmic lysate. Nuclear lysates were prepared from nuclei purified by sucrose gradient centrifugation. In brief, 1 x 10^{8} BC3 cells were washed in PBS and resuspended in 4 mL of ice-cold sucrose buffer A (0.32 M sucrose, 3 mM calcium chloride, 2 mM magnesium acetate, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1% Tween-20, 10 mM TRIS [pH 8.0], 0.1 mM sodium vanadate, aprotinin at 5 µg/mL, leupeptin at 5 µg/mL, pepstatin at 5 µg/mL, and Pefabloc at 150 µg/mL). Cells were transferred to a dounce homogenizer and broken with 6 strokes of pestle B.

CHAPTER III

RESULTS

K-cyclin Can Activate NF-κB-dependent Transcription

CyclinE/cdk2 and p300 assemble on NF-κB responsive genes and inhibit its transcriptional activity (Perkins et al. 1997). Additionally, inhibition of NF-κB regulated gene activity in multiple KSHV infected cell lines leads to apoptosis (Keller et al. 2000). Browning lab unpublished data suggest that K-cyclin can activate NF-κB dependent transcription in a dose-dependent manner, and we sought to duplicate that result. To assess the effect of K-cyclin on the transcriptional activity of NF-κB, we used transient transfection with a model NF-κB responsive reporter gene, NF-κB-Luc, containing five NF-κB consensus binding motifs. NF-κB Luc was cotransfected with pBKCMV/K-cyclin or the corresponding empty vector into the 293H cells. As previously shown, K-cyclin induced transcription of the NF-κB reporter construct. The constructs were co-transfected with a control reporter construct containing only a minimal promoter region, TATA-Luc. The K-cyclin-mediated transcriptional enhancement was abolished in cells containing the MRAIL mutants. The fold activation over that of control pBKCMV was approximately seventeen units above the baseline (**Figure 6**). These data suggest that K-cyclin induces transcription specifically through the NF-κB DNA binding consensus sequence.

MRAIL Mutants Can Be Translated into Full Length K-cyclin Protein

The MRAIL motif is a conserved protein-docking site in the N-terminus of both cellular and viral cyclins (**Table 1**). Its corresponding site is present in several proteins, including p105 and its splice product p50, c-Rel, Rel-B and p300 (**Table 2A**) in addition to putative phosphorylation sites (**Table 2B**). To determine whether the MRAIL-RXL interaction was essential for activation of NF-κB dependent transcription, we first generated single amino acid mutants of K-cyclin's MRAIL motif. We produced oligos matching K-cyclin's nucleotide sequence that included a wobble codon at the appropriate site. Using PCR and site-directed mutagenesis, we generated alanine mutants of the

amino acid residues comprising the MRAIL motif. This allowed a change in the amino acids deemed most important for compromise of the MRAIL protein-docking site, position 50's methionine, 51's arginine, 53's leucine and 57's tryptophan. In addition, we generated a mutant with a double alanine mutation at positions 50 and 54 and a triple mutant at position 50, 54 and 57. To characterize each mutant, each plasmid was sequenced for verification of the mutation and then the protein was *in vitro* transcribed and translated (**Figure 7**). We were able to show that each protein though mutated did produce full-length protein similar to that of wild type K-cyclin.

MRAIL Protein Docking Site Mediates K-cyclin Activation of NF-κB-dependent Transcription

To assess the effect of the MRAIL docking site on NF-κB dependent transcription, we co-transfected the NF-κB-Luciferase plasmid along with pBKCMV K-cyclin plasmid, into 293H cells. We were able to show that each mutant in a step-wise manner was sufficient to decrease the K-cyclin affect on the reporter construct. We graph these results next to the control plasmid and the previously shown wild-type K-cyclin affect. We were able to show an approximately seventeen-fold difference between K-cyclin and a mutant containing even one alanine in the MRAIL protein docking site. Furthermore, we show that there is not an additive affect with a further mutagenesis of the protein-docking site in the form of a double or triple mutant (**Figure 8**). These data suggest that K-cyclin's MRAIL motif mediates its effect on NF-κB dependent transcription.

LANA and K-cyclin Bind in KSHV-infected Cells

While its primary role involves episomal maintenance, LANA also functions as a transcriptional regulator in a variety of cellular and viral promoters, including its own (Lim et al. 2001, Renne et al. 2001, Garber et al. 2002, Verma et al. 2004). As K-cyclin and LANA are both transcribed from a single promoter, are expressed at the same time in the viral life cycle and are both found in the nucleus, we sought to determine whether they physically interact. Because both proteins are nuclear, the logical step was to extract the BC3 nuclei and look for an association. Using the nuclear lysate from BC3 cells

infected with KSHV, we used K-cyclin to immunoprecipitate a fraction of nuclear LANA at endogenous levels (**Figure 9**). Additionally, the fraction of LANA that associates with K-cyclin, in addition to being nuclear, is also akin to the fraction of LANA with the greatest amount of post-translational modifications compared to the total LANA available in the cell (**Figure 9, lane 1**).

LANA and K-cyclin Bind in Transfected Cos-7L Cells

The association between K-cyclin and LANA was shown at endogenous levels in KSHV infected BC3 cells. Because we do not have data to suggest that these proteins directly bind, we must investigate the nature of this association. To determine if this association requires other viral proteins, we expressed both proteins in a non-virally infected cell line, Cos-7L. By transfecting pcDNA 3.1 LANA and pBKCMV K-cyclin into Cos-7L cells, we were able to get expression of both proteins (**Figure 10A**). Again using K-cyclin as bait, we were able to immunoprecipitate LANA from whole cell lysate (**Figure 10B**). These data suggest that the K-cyclin and LANA complex may require other proteins to interact, but they are not viral in origin.

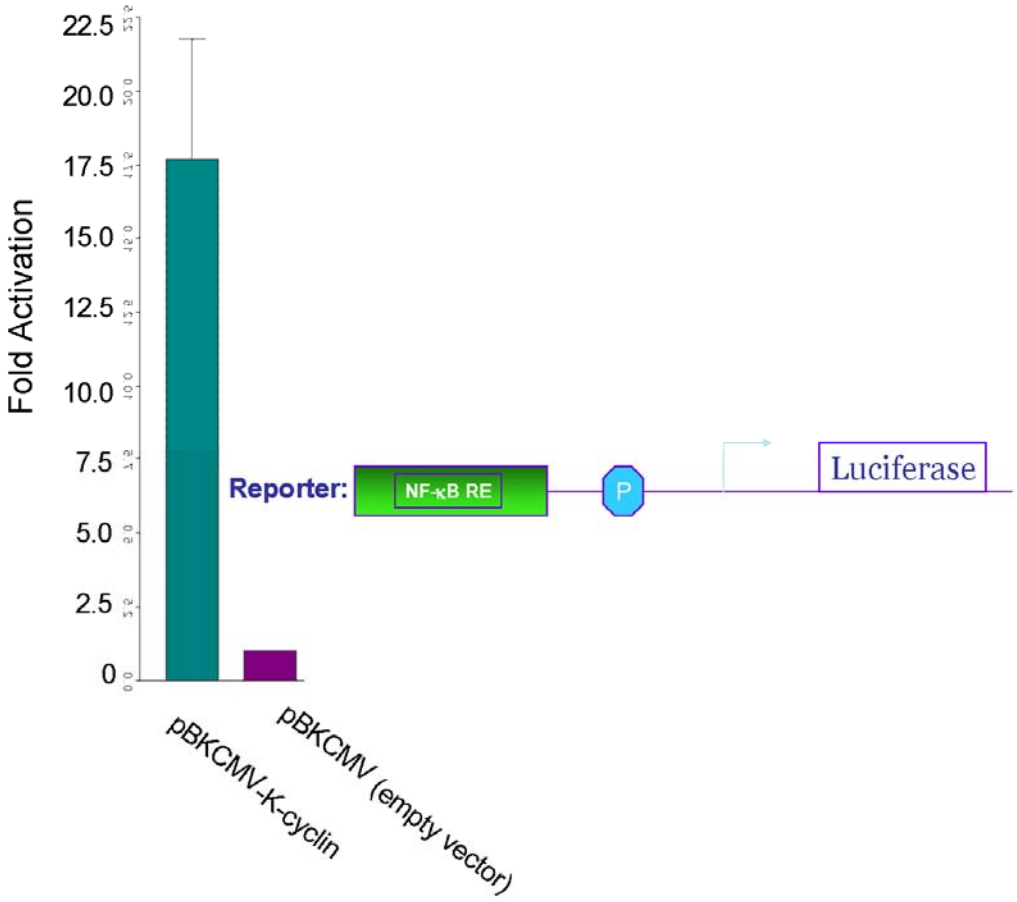

Figure 6**. K-cyclin Can Activate NF-kB Dependent Transcription. (A)** Kcyclin's action on the NF-kB recognition element was assessed by reporter assay.
The reporter plasmid contained five copies of the NF-kB recognition element in sequence along with a promoter tied to a luciferase gene. **(B)** pBKCMV, an empty vector, was compared to pBKCMV-K-cyclin, to determine whether the expression of the reporter gene was upregulated. When K-cyclin is present, NFkB- dependent transcription was increased approximately seventeen fold over the vector alone.

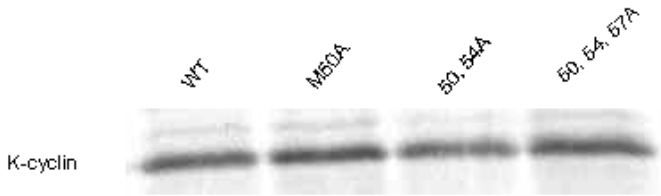

Figure 7. **MRAIL Mutations Make Full Length K-cyclin Protein.** To investigate the role of the MRAIL motif in K-cyclin activation of NF-| Bdependent transcription, we made mutations in the proteins. At position 50, the methionine was changed to alanine (K- cyclin M50A). We added a change of leucine at position 54 to the original methionine to alanine mutation and created a double mutant (K-cyclin 50, 54A). Finally, the tryptophan at position 57 was changed to alanine to create a triple mutant (K-cyclin 50, 54, 57A). Each of the proteins were *in vitro* transcribed and translated, to ensure that the mutations did not compromise the protein expression. We found that each of the proteins were expressed at the same level regardless of mutation.

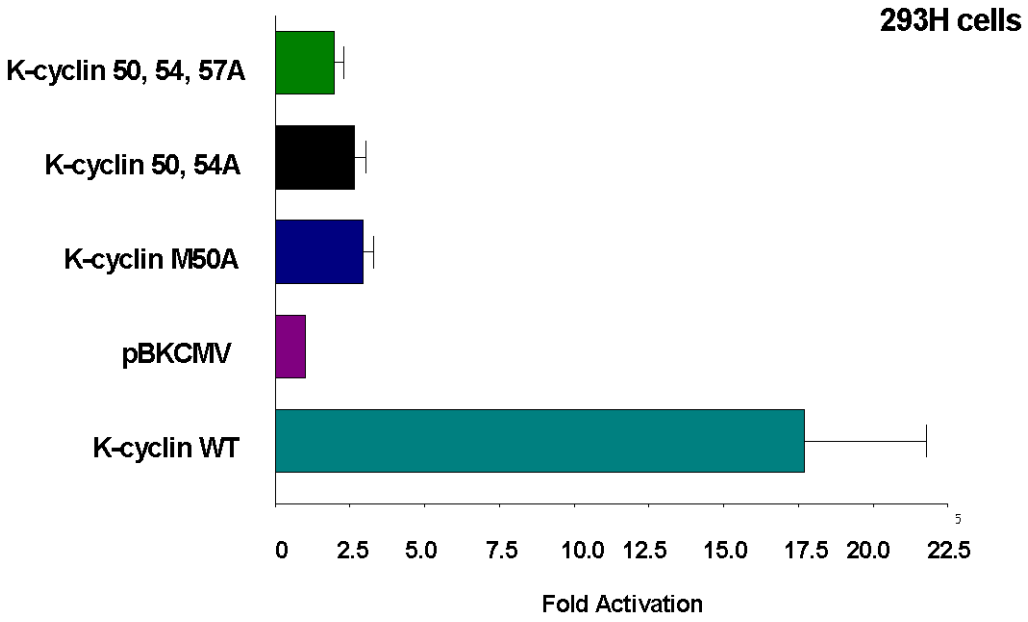

Figure 8. **MRAIL Mutants Reduce K-cyclin Activation of NF-┤ B-Dependent Transcription.** Based on the earlier findings that K-cyclin activated NF-┤ Bdependent transcription, we used the same assay to determine whether the MRAIL motif was important in this activation. We were able to show that Kcyclin activation was significantly higher than the empty vector (pBKCMV). Furthermore, when the K-cyclin mutants were added, each one reduced the activation over ten-fold. Additionally, there was no additive effect given by the double or triple mutants.

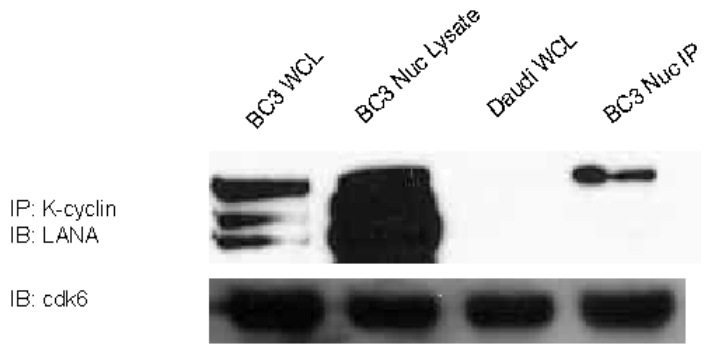

Figure 9. **K-cyclin Can Immunoprecipitate LANA from BC3 cells.** BC3 cells are a KSHV infected B cell line. Lane 1 shows the amount of LANA in BC3 cells. Lane 2 shows the amount of LANA available in BC3 nuclei. LANA can be immunoprecipitated by K-cyclin from BC3 cells as evidenced in lane 4.

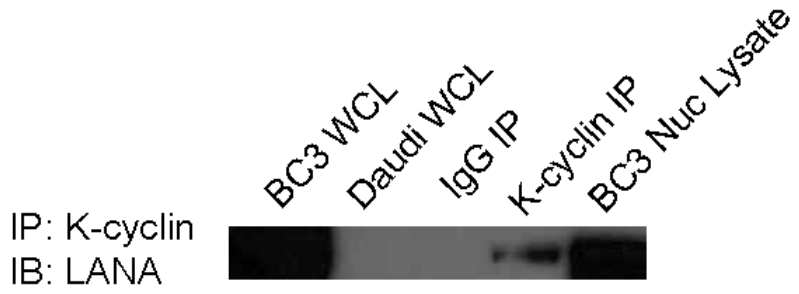

Figure 10. **LANA and K-cyclin, When Expressed in Cos-7L cells Physically Interact. (A)** pcDNA 3.1 LANA and pBKCMV K-cyclin were transfected into Cos-7L cells. The top panel is lysate (as labeled) probed with LANA antibody. The bottom panel is a western blot showing K-cyclin expression. The positive control is lane 1, BC3 whole cell lysate and the negative is lane 2, daudi cell lysate. **(B)** The transfected lysate was immunoprecipitated with K-cyclin and then probed for LANA. LANA and K-cyclin can co-immunoprecipitate in non-virally infected cells and without the expression of any other viral proteins. Lane 1 is BC3 whole cell lysate and lanes 2 and 3 are transfected Cos-7L lysate immunoprecipitated with sheep IgG and sheep K-cyclin antibody respectively.

CHAPTER IV

DISCUSSION

Introduction

Cellular and viral cyclins are emerging as multifunctional proteins. In addition to their roles as cell cycle regulators, data show that cyclin E, for example, is directly involved in transcription and licensing for DNA replication through its protein-protein interactions. In this case, K-cyclin, a viral cyclin mimic, is characterized by its unique protein-protein interactions including partnering with cdks 2, 4 and 6 (Godden-Kent et. al 1997). The literature provides definite evidence that K-cyclin is involved in cell cycle deregulation, evading apoptosis and DNA replication in addition to suggestive evidence and implications that it may play a role in transcription (**Figure 4**). The initial observation that K-cyclin associated with transcriptionally active chromatin opened a new realm of possibilities for a viral cyclin. The follow-up experiments with K-cyclin/cdk6 being constitutively active throughout the cell cycle and phosphorylating p300 correlate with the other data and suggest that K-cyclin may be implicated in transcription (**Figure 4**, Van Dross et al. 2005, unpublished data). Unpublished data from the Browning lab showed that K-cyclin could activate NF-κB dependent transcription, and we repeated those results in this report (**Figure 6**). The experiments discussed here look at the role of K-cyclin in one signaling cascade that p300 is a part of, NF-κB. Cyclin E binds to p300 within the carboxyl terminus only if complexed with cdk2 and if derived from a cellular source (Perkins et al. 1997). In contrast, our unpublished data shows that K-cyclin binds to p300 in the absence of cdk6, whether generated by *in vitro* transcription/translation or in bacteria. Moreover, because K-cyclin binds across multiple p300 epitopes, these regions could be in turn rendered susceptible to phosphorylation by K-cyclin/cdk6 complexes. Furthermore, phosphorylation of p300 may affect the binding of other transcriptional co-activator proteins required to activate NF-κB-dependent transcription.

MRAIL Protein Docking Site Mediates K-cyclin NF-κB Activation

Our studies sought to address the role of K-cyclin's protein-protein interactions on its non cell cycle related function. The work was based on our unpublished results that K-cyclin associates with the transcriptionally active fraction of chromatin and phosphorylates *in vivo* and *in vitro* transcribed and translated p300. Our data shows that K-cyclin can activate a reporter plasmid with an NF-κB DNA recognition element (**Figure 6**). In seeking a protein docking site to facilitate this interaction, we identified the MRAIL and RXL sites described by Schulman et al. whereby cdk2 recruits substrates through Cyclin A. We found that K-cyclin in addition to other cellular and viral cyclins contained this MRAIL motif in their N-terminal ends (**Table 1**).

Although our experiments have not yet elucidated the mechanism of this activation, there are two main possibilities. We know that K-cyclin is both nuclear and cytoplasmic, K-cyclin could be directly binding to the NF-κB sequence in DNA. On the other hand, K-cyclin/cdk6 complexes could phosphorylate proteins in the NF-κB signaling cascade (**Figure 5**, Van Dross et al 2005). These protein-protein interactions could happen with any proteins including p300. The p300 repressor domain, CRD1, is shown to be a mechanism for p300 to facilitate repression (**Figure 2**, Snowden et al. 2000). The cdk inhibitor, p21CIP/Waf1, binds to this p300 domain and reportedly activates by de-repression the NF-κB-dependent transcription. Although K-cyclin/cdk6 complexes are not inhibited by p21CIP/Waf1 *in vitro* and do not contain p21CIP/Waf1 in K-cyclin/cdk6 complexes derived from KSHV-infected cells, it is conceivable that phosphorylation of p300 by K-cyclin/cdk6 complexes could enhance p21CIP/Waf1 binding to the repressive domain. This binding would have the effect of enhancing NF-κB-dependent transcription. Our data suggest that K-cyclin activation of the NF-κB reporter is increased with the addition of exogenous p300, information we believe strongly supports this mechanism.

Several studies suggest that the cyclin subunit determines the substrate specificity of cyclin/cdk complexes. Studies evaluating how cyclin subunits determine substrate specificity using cyclins A and E suggest that important substrate binding regions reside in the carboxyl terminus of these cyclin proteins. We have identified a conserved sequence in the amino terminus of both cellular and viral cyclins that we believe is important in protein binding and subsequent phosphorylation (**Table 1**). This seven

amino acid sequence termed the MRAIL motif, has been shown to be important in substrate selection by cyclin A for its cdk2 kinase partner (Schulman et al. 1998). Additionally, a motif that may be important for phosphorylation by cdk6, a serine or threonine followed by a proline, has been identified in the K-cyclin/cdk6 kinase target, caldesmon (Cuomo et al. 2005). With all the potential docking and phosphorylation sites identified in proteins in the NF-κB cascade, we performed a mutational analysis to assess the role of K-cyclin's MRAIL motif on its ability to activate NF-κB. We have identified several proteins that coincide with K-cyclin's previously known or functions that contain these sites. This protein docking site may be important in binding and potential phosphorylation of K-cyclin target systems, cell cycle (**Table 3**) and DNA replication (**Table 4**). We sought to determine whether the MRAIL-RXL interaction was essential for activation of NF-κB dependent transcription. We have identified many NF-κB transcriptional regulators that have RXL motifs and potential phosphorylation sites (**Table 2**). There were at least two NF-κB subunits that did not contain RXL motifs. We believe that this is insignificant as NF-κB subunits act as a unit on DNA.

This data in concert with our observation that K-cyclin/cdk 6 complexes can activate NF-κB dependent transcription, led to the results reported here. The amino acids selected for mutation, the methionine at position 50, the leucine at position 54 and the tryptophan at position 57 were selected as they were the most important in the interaction between cyclin A and p107 (Schulman et al. 1998). We made K-cyclin mutants that contained mutations at position 50, a double mutant at positions 50 and 54 and finally a triple mutant with alanine substitutions at positions 50, 54, and 57. We were able to show that even with these mutations, the sequences created full-length protein (**Figure 7**). We found that the mutation of any single amino acid in the MRAIL motif is sufficient to disrupt the ability of K-cyclin to activate NF-κB luciferase reporter assay (**Figure 8**). One mutation alone was enough to significantly reduce NF-κB activation, with a double and triple mutation showing no additive effect. Mutagenesis of this MRAIL docking site may inhibit binding of the K-cyclin/cdk6 complex to its kinase target p300, thereby reducing its effect on activation of the reporter gene. Perkins et al. points out that the CRD1 binding motif of p300, can bind p21, in a distinct and different domain than the region where p300 binds to cyclin E/cdk2 complexes (Perkins et al. 1997). Consequently, the

K-cyclin MRAIL mutants, when bound, would not affect the ability of p21 to bind to p300. This cannot explain the reduction in NF-κB activation. Furthermore, p21 is an RXL protein that K-cyclin phosphorylates to bypass a G1 cell cycle arrest, and an affect on p21 could be the mechanism of repression (Jarviluoma et al. 2006).

NF-κB Activation By KSHV Latent Proteins

Keller et al. show that the PEL cell lines and clinical KSHV-infected PEL biopsy specimens contain constitutively activated NF-κB (Keller et al. 2000). Moreover, activated NF-κB appears to be essential for PEL cell survival because inhibition of this pathway results in apoptosis (Keller et al. 2000). v-FLIP uses NF-κB to block apoptosis by inhibiting the association of FADD and pro-caspases 8 and 10 (Thome et al. 1997). But, K-cyclin too can inactivate apoptosis through its phosphorylation of Bcl-2 (Ojala et al. 2000). While v-FLIP is an effective activator of NF-κB dependent transcription, it is only responsible for seventy percent of the activation associated with IL-6 (Guasparri et al. 2004). The three important latent gene products represent likely viral candidate genes that may constitutively activate the NF-κB pathway. LANA and v-FLIP have both been shown to activate IL-6 through interacting with NF-κB and AP-1 (An et al. 2002, 2003). A reasonable hypothesis based upon these findings is that KSHV-encoded gene products, including K-cyclin, LANA and v-FLIP, may be responsible for the constitutive NF-κB activation found in KSHV cell lines and biopsy specimens.

LANA and K-cyclin Bind in Both Viral and Non-Virally Infected Cells

Furthermore, because of the similarity in expression and implications for function, we questioned whether there was a further relatedness between the latent proteins. As K-cyclin and LANA are both transcribed from a single promoter, are expressed at the same time in the viral life cycle and are both found in the nucleus, we sought to determine whether they physically interact. By using both virally infected BC3 and transfected non-virally infected cells, we were able to show that K-cyclin associates with LANA in a complex that does not require any other viral proteins (**Figure 9 and 10B**).

We believe that the binding of these two proteins occurs to modulate one of their functions. While LANA does play a role in episomal maintenance, there is no literature to

support a role for K-cyclin in this process. LANA also functions as an inducer of DNA replication. In the TR region, there are two binding sites (LBS1/2), and when LANA is dimerized, it can occupy both (Fejer et al. 2003). When bound, a single LANA can bend DNA towards the major groove at approximately 57 degrees and when two bind, can bend it, at approximately 110 degrees (Wong et al 2005). In addition, K-cyclin phosphorylates Orc 1, a protein necessary for the formation of the pre-initiation complex. All of the Orc proteins (Orc1-6) can interact with LANA binding sequences in the TR region of the viral DNA. The interaction between the viral DNA and Orcs 1, 3, 4 and 5 are dependent on LANA's presence. With this in mind, we questioned whether the presence of K-cyclin could modulate LANA's affect on DNA replication but were unable to show a significant outcome (data not shown). The interaction between K-cyclin and LANA could suggest that LANA plays a role in cell cycle regulation. LANA can interact with proteins in the pRb/E2F pathway to stimulate cyclin E expression and protect cells from p16 INK4a induced cell arrest (An et al. 2005). It also can interact with β-catenin to influence the transcriptional action of GSK-3β (Fujimoro et al. 2005). LANA can also dramatically inhibit the transcriptional effect of p53 (Fribourg et al. 1999). But most of LANA's effect is through its role as a transcriptional regulator.

LANA has two mechanisms for regulating transcription: by binding to other transcription factors, or binding directly to DNA. LANA can bind to both c-Jun and Sp1 proteins to activate transcription of IL-6 and hTERT (An et al. 2002). On the other hand, LANA's DNA binding ability is shown when it regulates the viral promoter at the GC box/Sp1 recognition element despite the presence of DPE and TATA elements (Garber et al. 2001, Jeong et al. 2004). There is one published example of K-cyclin directly regulating the transcription of another protein, cyclin A. Although it does not phosphorylate pocket protein p107 or interact directly with E2F, transcription is facilitated through the E2F binding site in the cyclin A promoter and is dependent on cdk6 activity.

Because the proteins are made as a part of the same transcript, it is important to note that K-cyclin would also be present in the nucleus during LANA's transcriptional work. Furthermore, because these proteins were previously not known to associate, it is not surprising that it was never investigated as a part of these tasks. Since the Browning

lab was one of the first to consider a possible role for K-cyclin as a direct transcriptional regulator, it is possible that K-cyclin or K-cyclin/cdk complexes are involved in one or more of these cases. K-cyclin's primary role is to deregulate the infected cell's cycle, and regulating may be an additional mechanism to accomplish that goal. By binding to BRD4, LANA can regulate cyclin E transcription and release cells from a p16 induced G1 arrest (Ottinger et al. 2006). Further investigation into K-cyclin target genes could reveal that is a part of regulating the transcription of cell cycle regulators.

Conclusion

The NF-κB pathway is well-known for its role in transcriptional activation of several cytokine related genes, such as tumor necrosis factor (TNF)α, TNFβ, interleukin (IL)-1, IL-2, IL-6, and IL- 8 (Richmond, 2002). Therefore, the NF-κB-activating ability of K-cyclin/cdk6 complexes may have important implications for the natural history of KS and PEL. One of the unique features of Kaposi's sarcoma is the involvement of cytokines and growth factors in the autocrine and paracrine growth (Swanton et al. 1997). The constitutive kinase activity of K-cyclin/cdk6 complexes likely contribute to this activity. The findings described here strengthen the view that gammaherpesvirus-encoded cyclins and K-cyclin in particular, have broader functions than overcoming the cell cycle block and may function as transcriptional regulators. Therefore, we propose that chronic activation of the NF-κB-dependent transcriptional pathway by the KSHV latent gene products including LANA, K-cyclin and v-FLIP, may contribute to establishing latency, blocking apoptosis and the abnormal proliferative properties of KSHV-infected cells comprising tumors such as primary effusion lymphoma and Kaposi's sarcoma. Moreover, such chronic activation would set the stage for other genetic events that may ultimately result in cell transformation.

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